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tortuga refines Notch pathway gene expression in the zebrafish presomitic mesoderm at the post-transcriptional level

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Abstract

We have identified the zebrafish *tortuga* (*tor*) gene by an ENU-induced mutation that disrupts the presomitic mesoderm (PSM) expression of Notch pathway genes. In *tor* mutants, Notch pathway gene expression persists in regions of the PSM where expression is normally off in wild type embryos. The expression of *hairy/Enhancer of split*-related 1 (*her1*) is affected first, followed by the *delta* genes *deltaC* and *deltaD*, and finally, by another *hairy/Enhancer of split*-related gene, *her7*. In situ hybridization with intron-specific probes for *her1* and *deltaC* indicates that transcriptional bursts of expression are normal in *tor* mutants, suggesting that *tor* normally functions to refine *her1* and *deltaC* message levels downstream of transcription. Despite the striking defects in Notch pathway gene expression, somite boundaries form normally in *tor* mutant embryos, although somitic mesoderm defects are apparent later, when cells mature to form muscle fibers. Thus, while the function of Notch pathway genes is required for proper somite formation, the *tor* mutant phenotype suggests that precise oscillations of Notch pathway transcripts are not essential for establishing segmental pattern in the presomitic mesoderm.

Keywords: Somite; Segmentation clock; *her1*; *her7*; *deltaC*; *deltaD*; Cyclic genes

Introduction

The formation of somites establishes the initial segmental pattern along the anterior–posterior axis of vertebrate embryos. Somitic cells differentiate to form the axial skeleton, the dermis of the back, and striated muscle of the body wall and limbs; thus, segmentation of the embryonic mesoderm, in turn, establishes the metameric pattern of the adult body plan (reviewed in Keynes and Stern, 1988). In zebrafish, the bulk of the somite forms myotome (reviewed in Stickney et al., 2000). Bilateral pairs of somites appear as serially repeated blocks of cells formed from the presomitic mesoderm (PSM), a field of mesenchymal cells emerging from the tail bud on either side of the notochord. The formation of somites occurs in an anterior to posterior progression, with precise spatial and temporal regularity; within a given species, the number of somites formed and the time it takes to form a somite are fixed. In zebrafish embryos, a bilateral pair of somites form once every 20 min until 6 somites have formed and then once every 30 min until a total of 30–32 somites have formed (Kimmel et al., 1995). The clock and wavefront model suggests that two mechanisms cooperate in the PSM to generate somite boundaries at regular intervals in space and time (Cooke and Zeeman, 1976; for review, see Pourquie, 2003): the “clock”, a molecular oscillator with a period equal to the time it takes to form a somite, which coordinates groups of cells into presumptive somites; and the “wavefront”, a maturation gradient, which provides cells with positional information for proper boundary placement.

In recent years, evidence has emerged which provides insight into the molecular nature of the “clock” and the “wavefront”. The maturation wavefront in the PSM is regulated by FGF (Dubrulle and Pourquie, 2004; Dubrulle et al., 2001; Sawada et al., 2001) and Wnt (Aulehla et al.,

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2003) signals, which keep cells in an undifferentiated state in the posterior PSM, and by retinoic acid signaling (Diez del Corral et al., 2003; Moreno and Kintner, 2004; Vermot et al., 2005), which promotes differentiation in the anterior PSM. Mutational analyses and gene expression studies indicate that the Notch and Wnt signaling pathways are important components of the vertebrate segmentation clock (reviewed in Giudicelli and Lewis, 2004; Rida et al., 2004). The first molecular evidence of a clock in the PSM was the discovery of the dynamic expression pattern of chick *chairy1* (Palmeirim et al., 1997), a gene activated in response to Notch signaling. *chairy1* is expressed in waves, which move through the PSM in a posterior to anterior progression once every 90 min, the time it takes to form a somite in chick embryos (Palmeirim et al., 1997). Since the discovery of oscillating *chairy1* expression, cyclic Notch pathway genes have been identified in all vertebrate species examined, including *hairy*/*Enhancer of split*-related genes in chick (Leimeister et al., 2000; Palmeirim et al., 1997), mouse (Bessho et al., 2001), *Xenopus* (Li et al., 2003), and zebrafish (Gajewski et al., 2003; Holley et al., 2000; Oates and Ho, 2002; Sawada et al., 2000; Sieger et al., 2004), *lunatic fringe* in chick (Aulehla and Johnson, 1999; McGrew et al., 1998) and mouse (Forsberg et al., 1998), and *deltaC* in zebrafish (Jiang et al., 2000; Oates et al., 2005). Further evidence supporting a role for Notch signaling in the segmentation clock mechanism has emerged from mutational analyses. In zebrafish, mutations in and/or depletion of *beamter/deltaC* (S. A. Holley and J-Y. Jiang, personal communication), *deadly seven/notch1a* (Holley et al., 2000), and *after eight/deltaD* (Holley et al., 2002) exhibit disruptions in cyclic gene expression and in posterior somite formation (Holley et al., 2002; Oates et al., 2005; van Eeden et al., 1996, 1998), and simultaneous disruption of two *hairy/enhancer of split*-related genes, *her1* and *her7*, impairs somite formation along the entire anterior–posterior axis (Henry et al., 2002; Oates and Ho, 2002). Mice homozygous for null mutations in various Notch pathway components (including *Notch* and *Dll1*) exhibit defects in cyclic gene expression and somite formation (Conlon et al., 1995; Greco et al., 1996; Hrabe de Angelis et al., 1997). In addition to Notch pathway components, two genes encoding Wnt antagonists, *axin2* and *nkd1*, exhibit oscillating expression in the mouse PSM (Aulehla et al., 2003; Ishikawa et al., 2004), and mice homozygous for a hypomorphic mutation in *wnt3a* exhibit defects in cyclic gene expression and somite formation (Aulehla et al., 2003; Greco et al., 1996). Zebrafish embryos deficient in *receptor tyrosine phosphatase-psi* function, a gene implicated in Wnt signaling, have abnormal cyclic gene expression and somitic defects (Aerne and Ish-Horowicz, 2004).

It has been suggested that a negative feedback loop involving *hairy/Enhancer of split*-related transcriptional repressors (*hairy/hes/her* genes) constitutes a mechanism for oscillating gene expression in the vertebrate PSM (Dale and Maroto, 2003; Hirata et al., 2002; Lewis, 2003). In cell culture experiments, mouse Hes1 protein oscillates in 2 h cycles upon serum stimulation; oscillations are dependent upon the dynamic regulation of *hes1* mRNA and Hes1 protein, indicating that an auto-regulatory feedback loop generates the Hes1 oscillations (Hirata et al., 2002). A mathematical model suggests that an auto-inhibitory feedback loop involving Her1 and Her7 can describe a mechanism for the zebrafish somitogenesis oscillator (Lewis, 2003; reviewed in Pourquie, 2003). Computer simulations of this model demonstrate that a simple Her1/Her7 feedback loop is capable of generating and sustaining oscillations in gene expression, provided that delay times for transcription and translation are long relative to decay rates of mRNA and protein (Lewis, 2003). The strength of this model is that it can be used to make predictions about the consequences of disrupting various parameters in the system. For example, knock-in mice expressing a mutant Hes7 with a longer half-life but normal repressor activity exhibit severe disruptions in segmentation and cyclic gene expression after a few normal cycles (Hirata et al., 2004). Importantly, the specific timing and pattern of these disruptions fits remarkably well with mathematical predictions of Lewis' auto-inhibition model (Hirata et al., 2004).

We are taking a genetic approach in an effort to understand the regulation of cyclic genes during vertebrate segmentation. Here, we describe an ENU-induced mutation in the zebrafish *tortuga* (*tor*) gene that disrupts oscillating mRNA expression in the PSM; in addition to waves of cyclic gene expression, mutant embryos exhibit low levels of ectopic expression where expression is normally off. Disrupted expression is apparent for *her1* transcript at the beginning of segmentation; other cyclic genes are subsequently disrupted at later stages of segmentation. Using intron-specific *her1* and *deltaC* probes to detect newly transcribed and/or unprocessed transcript, we observed no expression difference between *tor* mutants and wild type siblings, suggesting that the transcriptional regulation of these genes is normal in *tor* mutant embryos and that the persistent expression represents accumulation of processed transcripts. In spite of the disruption in cyclic gene expression, *tor* mutants establish anterior–posterior polarity in the somitic mesoderm and initially form normal somite boundaries. However, defects in somite boundary maturation and myofiber development appear at later stages of somite differentiation.

Materials and methods

Zebrafish stocks and husbandry

Adult fish strains were kept at 28.5°C on a 14-h light/10-h dark cycle. Embryos were obtained from natural spawnings or in vitro fertilizations and were staged according to Kimmel

et al. (1995). One *tortuga* (*tor*) mutant allele, *b644*, was isolated in a screen designed to identify mutations that disrupt segmental gene expression. Methods for the screen have been described previously (Henry et al., 2002). In *tor* mutant embryos, it appears that cyclic gene transcripts are slow to degrade in the PSM; based on this observation, we named the mutation *tortuga* (Spanish for tortoise). The phenotypes described for *tor* mutants were present in ratios expected for Mendelian inheritance of a recessive mutation. We observed frequencies of 0.245:0.754 (mutant:wild type, $n = 3584$, $\chi^2 = 0.334$, $P > 0.5$) for the morphological phenotype and 0.249:0.751 (mutant:wild type, $n = 790$, $\chi^2 = 0.0016$, $P > 0.95$) for the *her1* expression phenotype.

In situ hybridization

Whole mount in situ hybridization was performed as previously described (Jowett, 1999). The *her1* intronic probe binds to the third intron of the *her1* transcript and was generously provided by M. Gajewski; the probe was hybridized at 55°C as previously described (Gajewski et al., 2003). The *deltaC* intronic probe binds to the fourth intron of the *deltaC* transcript and was generously provided by A. Oates; the probe was hybridized at 60°C as previously described (Oates et al., 2005). All other probes have been described previously and are referenced within the text. For *papc* expression (Figs. 3O and P), we observed one to two more stripes in mutant embryos than in wild type embryos; however, the total number of stripes observed was dependent upon the time of the coloration reaction. When the reaction was developed to near-saturation or saturation (~3 h), we observed an average of 3.7 ± 0.5 stripes in pre-sorted wild type embryos ($n = 20$) and 4.9 ± 0.5 stripes in pre-sorted mutant embryos ($n = 20$) at the 18 somite stage. In addition, we examined *papc* expression in unsorted embryos obtained from three separate clutches ($n = 55$) at the 12 somite stage; after 3 h of coloration, 40 embryos exhibited 3 to 4 stripes of expression, and 15 embryos exhibited 5 to 6 stripes, a ratio very similar to that expected for Mendelian segregation. All other expression patterns shown were observed in at least 10 mutant and 40 wild type embryos obtained from at least two separate $tor^{+/-}$ mating pairs. Color development for a given probe (Figs. 1–3, and Supplemental Fig. 1) was done strictly in parallel to enable comparisons between wild type and mutant panels. To estimate the level of ectopic mRNA expression in *tor* mutant embryos, we examined *deltaC* and *her1* staining intensity after different times of color detection (Supplemental Fig. 1). The intronic probes were detected with BM Purple (Roche) and color was developed for 5–7 h at room temperature. All other probes were detected with NBT-BCIP (Roche) and developed for 1–1.5 h at room temperature, depending upon the probe, or as indicated in Supplemental Fig. 1 and above for *papc*. Following probe detection, embryos were mounted as described previously (Melby et al., 1997) and photographed on a Zeiss Axioplan2 using an Axiocam digital camera.

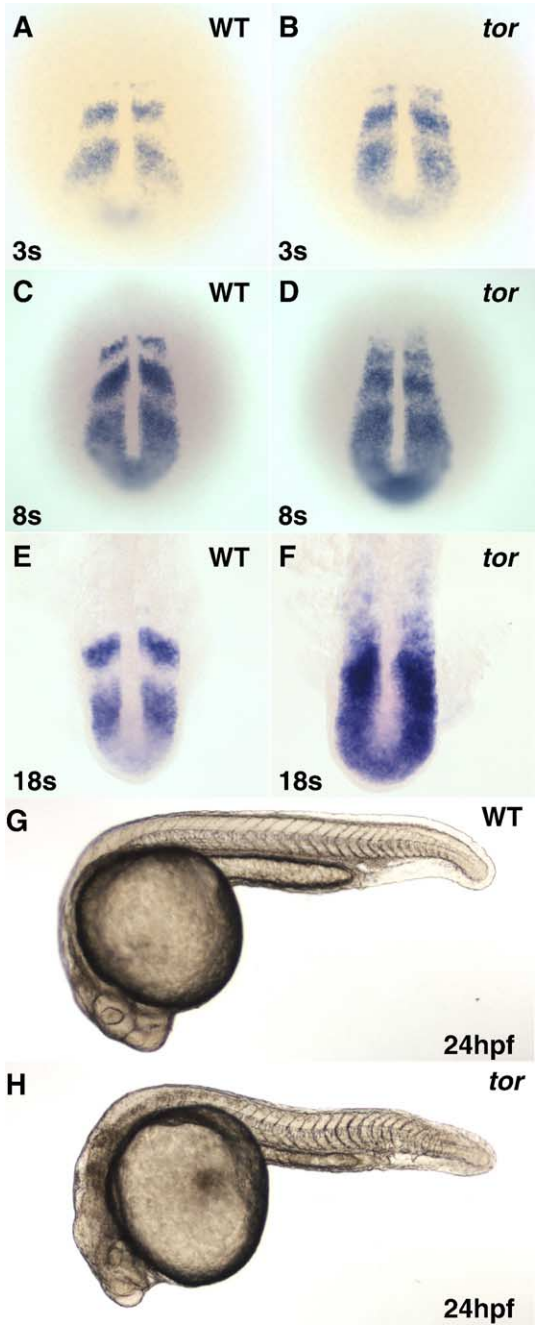

Fig. 1. Oscillating *her1* mRNA expression is disrupted in *tortuga* mutants, but somite boundary formation occurs normally. In situ hybridization in wild type (A, C, E) and *tor* mutant (B, D, F) embryos indicates that *her1* is misexpressed in *tor* mutant embryos from the beginning of segmentation, and defects get progressively worse as segmentation progresses. Panels are dorsal views, anterior top, with the somite stages indicated in lower left and phenotype indicated in the upper right of each panel. (G, H) Morphological examination shows that *tor* mutant embryos have segmental pattern, although posterior somites appear more U-shaped in *tor* mutant embryos compared to the wild type chevron shape. Other defects apparent in this view are head necrosis and reduced posterior yolk. Panels are lateral views of embryos at 24 hpf.

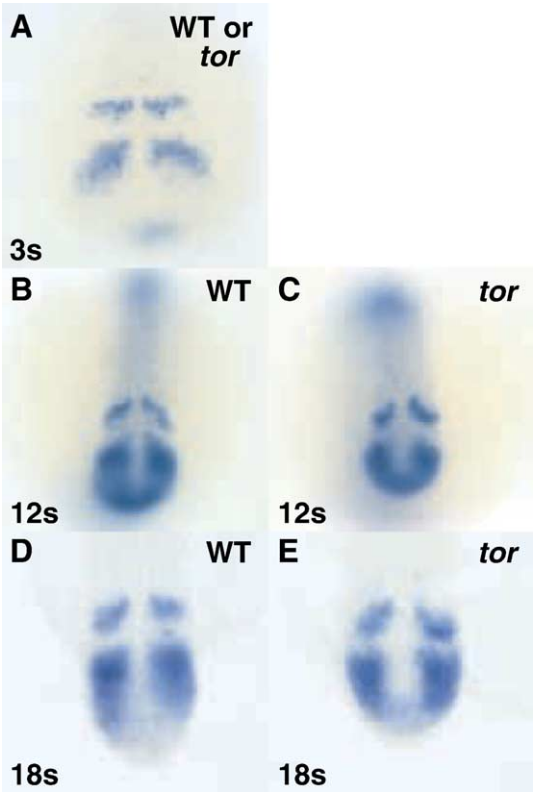

Fig. 2. *tortuga* regulates *her1* at the post-transcriptional level. Embryos were hybridized with an in situ hybridization probe that binds the third intron of *her1* and thus detects nascent and/or unprocessed transcript. At the 3 somite stage, mutant embryos in a clutch derived from $tor^{+/-}$ parents cannot be distinguished from wild type siblings (A). At 12 and 18 somite stages, *tor* mutant embryos were sorted from their wild type siblings by morphological criteria before hybridization; again, no differences in the pattern of *her1* transcription in wild type and *tor* mutant embryos are observed (B–E). Panels are dorsal views, anterior top.

Immunohistochemistry

F59 (monoclonal anti-slow myosin heavy chain, generously provided by Frank Stockdale) was used at 1:20 dilution (Devoto et al., 1996). Monoclonal anti-β-catenin was used at a 1:200 dilution to mark the cortex of all cells (Topczewska et al., 2001). Primary antibodies were detected using Alexa fluor 488-conjugated (for β-catenin) or peroxidase-conjugated (for F59) anti-mouse secondary antibody at a 1:200 dilution. After staining, embryos were mounted and photographed on a Zeiss confocal microscope or on a Zeiss Axioplan2 with an Axiocam digital camera. The contrast of original β-catenin-stained images was inverted in Adobe Photoshop so that cells are outlined in black.

Live embryo photography

Live embryos were mounted between bridged coverslips in embryo medium containing 0.004% tricaine and photographed on a Zeiss Axioplan2 with an Axiocam digital camera.

Results

A mutation in the tortuga gene disrupts her1 expression but does not disrupt somite boundary formation

The $tortuga^{b644}$ allele is an ENU-induced mutation recovered in a haploid-based genetic screen designed to identify genes that disrupt oscillating *her1* gene expression (screen described in Henry et al., 2002). In wild type embryos, *her1* mRNA is expressed in an oscillating pattern: Waves of gene expression initiate posteriorly and move anteriorly through cells of the PSM, but at fixed points in time, the expression appears as two to three distinct stripes (Figs. 1A, C, E; Holley et al., 2000; Sawada et al., 2000). In *tor* mutant embryos, dark stripes of gene expression are apparent, but lower levels of ectopic expression persist between stripes where transcripts are normally not detected (Figs. 1B, D, F). Thus, *her1* expression appears to oscillate in *tor* mutant embryos, but the difference between the "on" and "off" phases is diminished, resulting in what we term as damped oscillations. The defects in *her1* expression are first detected in *tor* mutant embryos at the beginning of segmentation (10.5 h post-fertilization [hpf], data not shown), and ectopic *her1* expression intensifies as embryos develop (compare Figs. 1B, D, F).

tor mutant embryos develop through cleavage, gastrulation, and early segmentation stages, with no obvious morphological defects. Mutant embryos survive up to 72 hpf; however, overall size and morphology suggest that development arrests at approximately 40 hpf. The first morphological defect is head necrosis, which is readily distinguished at 10 somites (14 hpf). At 24 hpf, *tor* mutant embryos have a reduced posterior yolk, slightly U-shaped posterior myotomes, and necrosis in anterior neural tissues (Figs. 1G, H). Later, they show uncoordinated twitching of the body, delayed heart development, lack of circulating blood cells, cardiac edema, and curvature of the posterior axis. Despite the *her1* expression defect, somite formation proceeds quite normally in *tor* mutant embryos.

tortuga refines the her1 expression pattern at a post-transcriptional level

Excess *her1* transcripts in *tor* mutant embryos could result from a delay in mRNA processing, prolonged transcription, and/or slower mRNA decay. To distinguish among these possibilities, we examined nascent and/or unprocessed *her1* transcripts in *tor* mutant embryos using a probe specific to the third intron of *her1*. The intronic probe reveals the expression domains of newly transcribed and unprocessed *her1* transcripts, and in wild type embryos, *her1* expression detected with the intronic probe is indistinguishable from the expression detected with an exonic probe (Gajewski et al., 2003). At 3 somites, *tor* mutant embryos cannot be distinguished from wild type siblings based upon the expression of *her1* nascent tran-

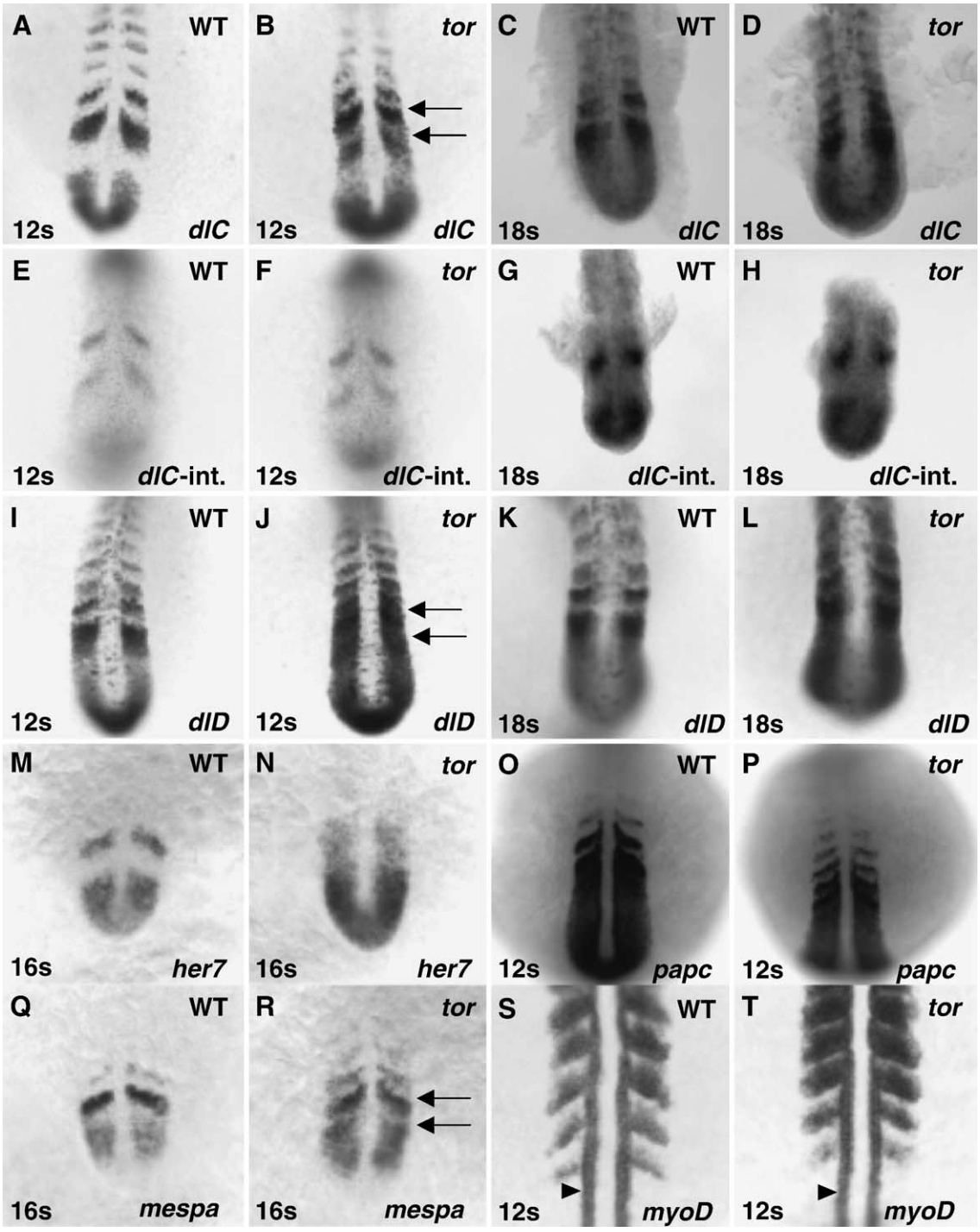

Fig. 3. *tor* mutant embryos exhibit distinct disruptions in the expression of Notch pathway genes and subtle disruptions in somite polarity. (A–D and I–N) Dynamically expressed Notch pathway genes, *deltaC (dlC)*, *deltaD (dlD)*, and *her7*, are misexpressed in *tor* mutant embryos in a manner similar to *her1*, with normal stripes and ectopic expression between stripes, although the timing of disruption is delayed relative to *her1*. Arrows in Panels B and J indicate ectopic expression in *tor* mutant PSM. (E–H) An intronic probe for *deltaC* was used to detect nascent and/or unprocessed transcript. At 12 and 18 somite stages, the pattern of *deltaC* transcription in *tor* mutant embryos appears normal (compare to exonic probe hybridization in Panels A–D). (O–T) Markers of somite polarity exhibit subtle disruptions in *tor* mutant embryos. In mutant embryos, *papc* expression is normal within the PSM, but expression is detected longer after somites form; after 3 h of coloration detection, we see 3 to 4 stripes of *papc* expression in wild type embryos (O) and 4 to 6 stripes in *tor* mutant embryos (P). The polarized expression of *mespa* in the presumptive anterior compartments is apparent in wild type and *tor* mutant embryos (Q, R); however, low levels of transcript are also detected in the posterior compartment of presumptive somites in mutant embryos (arrows in R). The posterior polarity of *myoD* is maintained in *tor* mutant embryos, although *myoD* stripes are not as distinct as in wild type, and some transcript is detected in the anterior compartment (S,T). *myoD* expression in slow muscle precursors (arrowheads in S and T) is normal in *tor* mutant embryos. Panels are dorsal views, anterior top. Genotype, stage, and probe are indicated on each panel.

script (Fig. 2A), even though they are readily distinguished by the expression of processed *her1* transcript at this stage (Fig. 1B). At 12 and 18 somite stages, *tor* mutant embryos were identified based on morphological defects, but the *her1* transcription pattern is still normal in *tor* mutants at these stages (Figs. 2B–E). The expression pattern revealed with the *her1* intronic probe indicates that the apparent damped oscillation of *her1* expression in *tor* mutants is due to persistence of processed *her1* transcript and not to prolonged transcription or delayed splicing. These data suggest that *tor* normally functions in the PSM to refine the *her1* expression pattern at the post-transcriptional level.

Notch pathway gene expression is disrupted in tortuga mutant embryos

The persistence of *her1* transcript in *tor* mutant embryos suggests that the mutation may disrupt a component of the segmentation clock. Alternately, *tor* could encode a gene that is specifically required for post-transcriptional *her1* mRNA processing but is not involved in the overall clock mechanism. We hypothesized that a mutation that disrupts a component of the segmentation clock would also disrupt the expression of other cyclic genes in the PSM.

In wild type embryos, the *delta* genes *deltaC* and *deltaD* are initially expressed in a broad domain in the posterior PSM; expression is refined in the anterior PSM into distinct stripes corresponding to the anterior (*deltaD*) or posterior (*deltaC*) half of the next two presumptive somites (Figs. 3A, C, I, K; Holley et al., 2000; Jiang et al., 2000). Like *her1*, *deltaC* has been described as a cyclic gene (Jiang et al., 2000), and although *deltaD* expression does not oscillate, it has been linked to the segmentation clock (Holley et al., 2000). In clutches of embryos from $tor^{+/-}$ parents, *deltaC* and *deltaD* expression is normal during the formation of the first five somites (a stage when *her1* expression is disrupted). Beginning at 5–6 somites (12 hpf), 25% of the embryos in a clutch exhibit subtle disruptions in *deltaC* and *deltaD* expression patterns similar to the disrupted expression of *her1* seen in *tor* mutant embryos: normal stripes with low levels of ectopic expression between stripes (data not shown). The level of ectopic expression increases as segmentation continues, and the difference between wild type and mutant expression patterns is obvious at 12 somites (15 hpf) and 18 somites (18 hpf) (Figs. 3A–D and I–L).

To determine if the *tor* mutation disrupts the cyclic expression of *deltaC* in the same manner as observed for *her1* (Fig. 2), we examined the pattern of *deltaC* transcription using a probe specific to the fourth intron of *deltaC*. This riboprobe is reported to detect a pattern in the PSM that closely resembles the pattern detected with the *deltaC* exonic probe, although the stripes detected with the intronic probe are notably less broad than the corresponding exonic signal (Oates et al., 2005). At 12 and 18 somite stages, the *deltaC* intronic probe does not reveal a difference in *deltaC* transcription between the wild type and *tor* mutant embryos (Figs. 3E–H), suggesting that the *tor* lesion disrupts *deltaC* expression at the post-transcriptional level.

The *hairy/Enhancer of split*-related gene *her7* is required for normal posterior somitogenesis (Gajewski et al., 2003; Henry et al., 2002; Oates and Ho, 2002) and is functionally redundant with *her1* and *deltaC* for anterior somite formation (Henry et al., 2002; Oates and Ho, 2002; Oates et al., 2005). As expected, *her7* is expressed in an overlapping pattern with *her1*, with the exception of the most anterior expression domain; at fixed points in time, *her7* is expressed in 1–2 PSM stripes instead of 2–3 stripes (Fig. 3M; Gajewski et al., 2003; Oates and Ho, 2002). Prior to mid-segmentation, *her7* expression is normal among progeny of $tor^{+/-}$ parents at stages before (1–10 somites; 10–14 hpf) or after (12 somites; 15 hpf) the morphological phenotype is apparent (data not shown). However, by 16 somites, stripes of *her7* transcript are not detected in mutant embryos, and instead, the transcript is expressed in a broad domain with expression persisting in regions where it is normally not detected in wild type embryos (Fig. 3N).

Taken together, these results indicate a general disruption in cyclic gene expression in *tor* mutant embryos; the *her1* gene expression pattern is the earliest pattern disrupted, and expression patterns of the other Notch pathway genes are subsequently disrupted 2 h (*deltaC* and *deltaD*) to 7 h (*her7*) after *her1* defects are apparent. The expression pattern revealed by the *deltaC* intronic probe indicates that the ectopic *deltaC* expression in *tor* mutant embryos is due to the persistence of processed transcript rather than a disruption in *deltaC* transcription. Thus, although *her1* appears more sensitive to *tor* disruption, the mutation affects the expression of at least two Notch pathway genes, *her1* and *deltaC*, in a similar manner.

The polarity of anterior and posterior somite compartments is subtly disrupted in tortuga mutant embryos

In the anterior PSM, presumptive somites are patterned into anterior and posterior compartments. The compartmentalization is visualized by the expression of genes such as *paraxial protocadherin* (*papc*) (Yamamoto et al., 1998) and *mespa* (Durbin et al., 1998; Sawada et al., 2000) in anterior compartments and *ephrinB2* (Durbin et al., 1998) and *myoD* (Weinberg et al., 1996) in posterior compartments. In zebrafish Notch pathway mutants, genes normally restricted to anterior or posterior compartments are instead expressed in broad domains that do not indicate polarity in the presumptive and formed somites (Holley et al., 2000, 2002; Jiang et al., 2000; van Eeden et al., 1996, 1998). Because anterior–posterior patterning is disrupted in Notch pathway mutants and Notch pathway gene expression is disrupted in *tor* mutants, we examined anterior–posterior patterning in *tor* mutant somites.

In wild type embryos, *paraxial protocadherin* (*papc*) is broadly expressed throughout the posterior PSM, but is refined in the anterior PSM into stripes marking anterior

compartments of presumptive somites (Yamamoto et al., 1998). Once a somite forms, *papc* transcript disappears rapidly and is detected only transiently in the most recently formed 1–2 somites (Fig. 3O; Yamamoto et al., 1998). In *tor* mutant embryos, polarized *papc* expression is established in the anterior PSM, but expression persists so that *papc* is detected in 3–4 formed somites (Fig. 3P; see Materials and methods). The extra stripes of *papc* expression are detected in *tor* mutant embryos as early as 10 somites. *mespa* encodes a basic helix–loop–helix transcription factor expressed during segmentation stages in bilateral stripes corresponding to the anterior compartments of the next one or two presumptive somites (Fig. 3Q; Sawada et al., 2000). In *tor* mutant embryos, *mespa* expression is indistinguishable from the wild type pattern until the 16 somite stage (17 hpf). At this stage, the expression pattern is slightly disrupted; *mespa* expression appears segmental in mutant embryos, but low levels of transcript are also detected in the presumptive posterior compartment (Fig. 3R). Subtle disruptions in the expression of *myoD*, which encodes a myogenic determination factor expressed in the posterior domain of formed somites, were detected as early as the 12 somite stage (15 hpf) in *tor* mutant embryos. While *tor* mutants display stripes of *myoD* expression, the anterior boundaries of the stripes are slightly expanded (compare Figs. 3S and T). Although the data indicate a slight disruption in anterior–posterior somite patterning in *tor* mutant embryos, the genes we examined still exhibit a clear polarity to their expression pattern, and the defects are quite subtle compared to the disrupted patterns that have been described for Notch pathway mutants.

In tor mutant embryos, somite boundary formation is normal, but somitic cell elongation is delayed

We hypothesized that disruptions in Notch pathway gene expression combined with the subtle defects in anterior–posterior polarity described above might cause defects in somite boundary formation in *tor* mutant embryos, so we examined individual cell morphology in forming and formed somites. During somite formation in wild type embryos, cells at the boundary align and undergo epithelialization, while cells in the middle of the somite remain mesenchymal (Figs. 4A, A″; Barrios et al., 2003; Henry et al., 2002). An examination of β-catenin expression (which marks cell membranes) reveals that boundary morphogenesis is normal in *tor* mutant embryos: Border cells in the newly formed and forming somites are arranged in neat rows and have a typical rectangular shape of epithelial cells (Figs. 4B, B″). As a somite matures, cells elongate between anterior and posterior somite boundaries and differentiate into myofibers. This elongation process is significantly delayed in *tor* mutant embryos; many cells in the anterior somites of mutant embryos are still round when compared to fully elongated cells at the same anterior–posterior position in wild type embryos (compare Figs. 4A′ and B′). Unlike the Notch pathway gene expression defects, the delay in muscle fiber elongation does not appear to be more severe in posterior

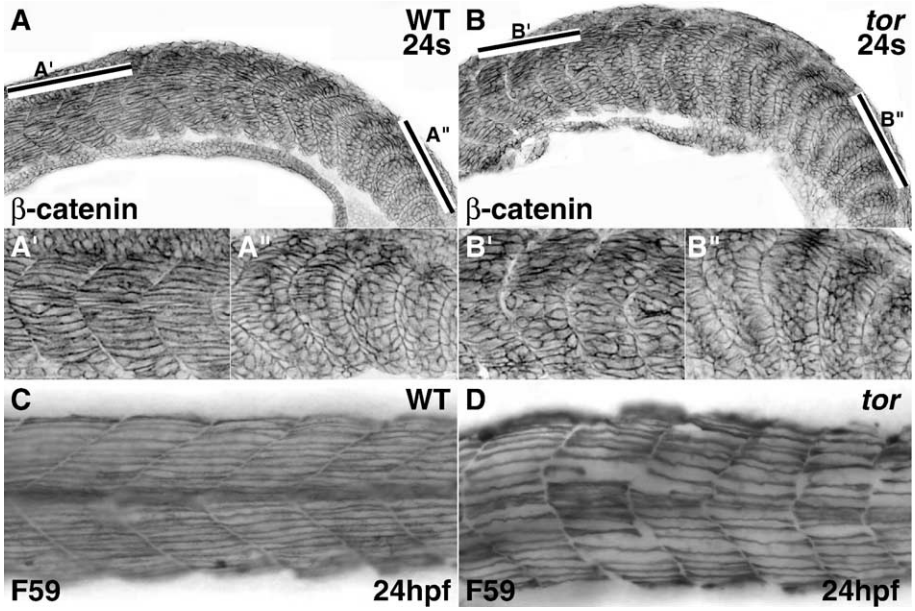

Fig. 4. Somite boundaries form normally, but defects appear later, as *tor* mutant somitic cells mature into skeletal muscle. Confocal micrographs of β-catenin expression show similar cubiodal morphology of cells at newly formed/forming boundaries in wild type (A, A″) and *tor* mutant (B, B″) embryos. In wild type embryos, myofibers in anterior somites have extended across the entire myotome (A′), while myofibers in anterior mutant somites have not completed elongation (B′). The bars in Panels A and B indicate the respective regions magnified in the prime and double prime panels. Slow muscle myosin expression (C, D) shows the abnormal morphology of slow muscle in *tor* mutant embryos; slow fibers in mutant embryos are irregularly spaced, do not extend in a straight trajectory, and boundaries between the myotomes are irregular. Panels are lateral views, anterior to the left. Genotype and stage are indicated on the panels.

somites when compared to anterior somites (Supplemental Fig. 2).

Muscle fiber organization is disrupted in tor mutant embryos

As described above, *tor* mutant embryos display defects in aspects of muscle fiber morphogenesis. Zebrafish have two main classes of muscle fibers, slow-twitch muscle and fast-twitch muscle (Devoto et al., 1996; Stickney et al., 2000). Slow muscle precursors are a specialized group of somitic cells that initially abut the notochord before migrating laterally through the somite to form superficial muscle fibers (Devoto et al., 1996; Stickney et al., 2000). Slow muscle precursors are the first cells in the somitic mesoderm to express muscle differentiation factors, such as *myoD*, and the first to elongate into fibers (Devoto et al., 1996; Stickney et al., 2000). The expression of *myoD* in slow muscle precursors is the same in wild type and *tor* mutant embryos (Figs. 3S and T, arrowheads), suggesting that early steps of slow muscle specification occur normally in *tor* mutant embryos.

To visualize slow muscle morphology, we examined slow muscle myosin expression. In wild type embryos, fibers are aligned in regularly spaced parallel arrays, individual fibers transverse the anterior–posterior extent of the myotome in a straight trajectory, and the boundaries between the myotomes have a characteristic chevron shape (Fig. 4C). Slow fibers in *tor* mutant embryos have an abnormal morphology; they are irregularly spaced, often do not extend in a straight trajectory, and boundaries between the fibers/myotomes are irregular (Fig. 4D). Although β-catenin expression revealed a delay in muscle fiber elongation at 24 somites (21 hpf) in *tor* mutant embryos, most cells expressing slow muscle myosin have completed elongation by 24 hpf. The expression of slow muscle myosin in *tor* mutant embryos does not indicate a defect in slow muscle differentiation at 24 hpf; however, there is clearly a defect in the general shape of the myotome and in the morphology of individual slow muscle fibers. The defects in slow muscle morphology become less severe as the somite matures and, unlike the Notch pathway expression defects, slow muscle morphology does not appear to be more severely disrupted in posterior somites than in anterior somites (Supplemental Fig. 2).

Discussion

The persistence of cyclic gene transcripts in tortuga mutants is due to abnormal post-transcriptional regulation

In wild type zebrafish embryos, the expression of the cyclic genes oscillates between two phases of expression within a 30-min period: an "on" phase, when the gene is transcribed, and an "off" phase, when the gene is not transcribed. In order for the expression patterns of *her1*, *her7*, and *deltaC* to be resolved into distinct stripes corresponding to the "on" and "off" phases of the oscillation, mRNA transcripts must be rapidly degraded. In situ hybridization studies using intronic probes for *her1* (Gajewski et al., 2003) and *deltaC* (Oates et al., 2005) indicate that the cyclic expression of these genes is regulated, at least in part, at the level of transcription. Transcriptional regulation has also been demonstrated with *her1* promoter analysis that shows that the dynamic expression of the *her1* gene is generated by differential transcriptional regulation (Gajewski et al., 2003). In addition, this promoter study also highlights the importance of post-transcriptional regulation, which leads to rapid degradation of *her1* transcripts and prevents their accumulation in the PSM (Gajewski et al., 2003). A mathematical model of the zebrafish segmentation clock also predicts that rapid mRNA degradation is a key factor in generating sustained oscillations of gene expression (Lewis, 2003). The differential expression of *her1* and *deltaC* transcripts detected using exonic versus intronic probes suggests that *tor* mutant embryos have a decreased rate of cyclic gene mRNA decay.

Although it is generally assumed that rapid mRNA degradation is an important component of cyclic gene regulation in the PSM, there have been few clues as to the mechanism of this regulation. One important piece of data comes from transgenic studies in *Xenopus*, which show that the segmental expression of *xhairy2* in the PSM is dependent upon rapid RNA decay and that a 25 bp motif in the 3′UTR of *xhairy2* is required for rapid mRNA turnover (Davis et al., 2001). This same motif was identified in the 3′ UTR of *hairy/enhancer of split*-related cyclic genes in mouse and chick, but it is not found in *her1* or *her7*, suggesting that mRNA degradation in the PSM is regulated differently for these genes. The *tor* mutant phenotype follows a typical Mendelian inheritance pattern for a recessive mutation indicating that tor^{b644} is likely a hypomorph or null allele. Thus, tor^{b644} may identify a gene that is normally involved in the degradation of cyclic Notch pathway transcripts in the zebrafish PSM. Based on our genetic linkage data, *her1* itself, as well as the linked *her7* gene, has been ruled out as *tor* candidate gene (data not shown).

Why is somite boundary formation normal in tortuga mutants?

Analyses of Notch pathway mutants (reviewed in Rida et al., 2004) and embryos overexpressing Notch pathway genes in the PSM (Hirata et al., 2004; Takke and Campos-Ortega, 1999) demonstrate that Notch signaling is required for somite boundary formation and for establishing anterior–posterior somite polarity. Based on these previous observations, we expected that the persistence of Notch pathway transcripts in *tor* mutants would lead to defects in

somite polarity and somite boundary formation. However, our data show that *tor* mutant embryos form normal epithelial somite boundaries (Fig. 4) and that somite polarity is maintained with only slight disruptions (Fig. 3). This finding was surprising because oscillating Notch signaling is a central component of current models for vertebrate segmentation (Giudicelli and Lewis, 2004; Lewis, 2003; Pourquie and Goldbeter, 2003). Our data show that there are still "peaks" and "troughs" of Notch pathway gene expression patterns in *tor* mutant embryos which might explain the observation of normal segmentation. Additionally, others have also observed normal somite formation in embryos with defects in cyclic gene expression. In zebrafish Notch pathway mutants, the most anterior somites form normally, even though cyclic gene expression is already disrupted at these stages (Jiang et al., 2000; van Eeden et al., 1996, 1998). Embryos deficient in *her1* function form normal posterior somites (Henry et al., 2002; Oates and Ho, 2002), but cyclic gene expression is disrupted in these embryos at the time when normal somites are forming. These data suggest that, while Notch signaling is a component of the segmentation mechanism, the precise oscillation of Notch pathway transcripts is not required for somite formation.

To better understand the observation of normal somite formation in *tor* mutants, we carefully examined the developmental onset and the nature of the disruptions in cyclic gene expression. During early segmentation, most cyclic gene expression patterns are still in place in *tor* mutant embryos. *her1* mRNA oscillations are damped, but other cyclic genes are expressed normally; in fact, *her7* expression is normal until 16 somites (17 hpf). Previous experiments testing the effects of *her1* and *her7* loss-of-function have shown that embryos that lack Her1 but maintain Her7 have defects in anterior somite boundaries, but posterior somite boundaries form normally (Henry et al., 2002; Oates and Ho, 2002). *her7* is expressed normally in *tor* mutants until 16 somites and could therefore compensate for the loss of *her1* mRNA oscillations during early to mid segmentation. However, posterior somites that form after *her7* mRNA oscillations are disrupted also form normally in *tor* mutant embryos. This finding suggests a distinction between the loss of Her1/Her7 function and the loss of proper *her1/her7* mRNA oscillations. Our data show that the transcription of *her1* continues to oscillate in *tor* mutants, suggesting that cells in the PSM are still exposed to a periodic increase in the level of *her1* transcript, even if processed transcript persists longer. Perhaps, these transcriptional "bursts" provide enough information for cells in the PSM to interpret the phase of the segmentation clock. Another possibility is that molecular oscillations are required early to initiate segmentation, but once metameric pattern is established, anterior somites are able to provide information about polarity and boundary position to more posterior somites. Finally, it is possible that the presence of ectopic Notch pathway transcripts in *tor* mutants does not necessarily lead to ectopic functional protein, an idea we discuss below.

Elevated her1 mRNA levels in tor mutant embryos do not cause a Her1 gain-of-function phenotype

While *her7* can compensate for a loss of *her1* function (except in the most anterior somites), in situ hybridization suggests that *her1* transcript is present at normal if not elevated levels rather than lower levels in *tor* mutant embryos. This expression pattern predicts a gain-of-function rather than loss-of-function *her1* phenotype; however, the *tor* mutant phenotype does not correspond to reported Her/Hes gain-of-function phenotypes. Overt segmentation defects are observed in zebrafish embryos overexpressing *her1* mRNA (Takke and Campos-Ortega, 1999) and in mice expressing stabilized Hes7 protein (Hirata et al., 2004). In addition to obvious disruptions in somite morphology, Her/Hes gain-of-function embryos have defects in somite polarity; genes that are normally expressed in either the anterior or posterior compartments are instead expressed in broad domains that do not indicate polarity in presumptive or formed somites (Hirata et al., 2004; Takke and Campos-Ortega, 1999). *tor* mutants do not exhibit morphological defects in somite formation, and the expression patterns for genes that mark anterior or posterior somite polarity are only slightly disrupted. We therefore hypothesize that the apparent increase in *her1* mRNA in *tor* mutants does not correspond to a large increase of functional Her1 protein. This result could be achieved if *her1* transcripts are not as efficiently translated in *tor* mutant embryos, or if accessory factors are necessary for Her1 function.

tortuga mutants have skeletal muscle defects

Although somite boundaries form normally in *tor* mutants, *tor* mutant myotomes are somewhat U-shaped as opposed to the normal chevron-shape. A class of zebrafish mutants with U-shaped somites (the *you*-type mutants) have reduced or missing slow muscle cells (van Eeden et al., 1996, 1998). Hedgehog signaling from the notochord specifies slow muscle (reviewed in Hughes, 2004), and many of the *you*-type mutants disrupt genes that are required for Hedgehog signaling or regulated by Hedgehog signaling (Karlstrom et al., 1999; Kawakami et al., 2005; Roy et al., 2001; Schauerte et al., 1998; Varga et al., 2001). In *tor* mutants, Hedgehog-dependent specification of slow muscle is normal, as indicated by normal expression of *myoD* and slow muscle myosin. However, other steps may be affected. For example, we have recently shown that slow muscle elongation and migration is required for timely elongation of fast fibers (Henry and Amacher, 2004), and here, we show that muscle fiber elongation is delayed in *tor* mutants (Fig. 4). Thus, although *tor* mutants are not defective in slow muscle specification or differentiation, the U-shaped somites, delayed fiber elongation, and irreg-

ular myotome boundaries suggest that there may be defects during the maturation process. These muscle defects indicate that, in addition to regulating cyclic gene expression in the PSM, *tortuga* might also function during muscle maturation. Alternatively, the muscle defects could be a downstream result of abnormal Notch pathway gene expression in the PSM. However, unlike the Notch pathway gene expression phenotype, the muscle fiber defects do not appear to be more severe in posterior somites (Supplemental Fig. 2). This observation supports the former explanation suggesting a more direct role for *tortuga* during muscle maturation.

A model for tortuga function during somitogenesis

Our data show that *tor* mutant embryos have increased levels of *her1* and *deltaC* mRNA in the PSM, but the transcriptional oscillation of these genes appears normal. It has been suggested that rapid mRNA degradation is a key factor in generating and maintaining oscillating gene expression in the PSM (for review see Giudicelli and Lewis, 2004). Because *her1* and *deltaC* transcription is normal in *tor* mutants, we propose that the *tor* lesion identifies a gene that promotes the degradation of mRNA transcripts in the PSM. The *tor* lesion might disrupt or delay a general RNA decay mechanism. As a result, mRNA transcripts that are normally degraded rapidly (e.g., cyclic genes) exhibit disrupted expression patterns, while transcripts that are not rapidly degraded are not overtly disrupted in *tor* mutant embryos. Alternately, the *tortuga* gene may act as part of the segmentation clock to specifically degrade cyclic Notch pathway transcripts in the PSM. For example, a model presented in a recent review suggests that Her proteins act to repress their own promoters to generate sustained oscillations in gene expression; rapid degradation of *her1* mRNA is a key component of this model (Giudicelli and Lewis, 2004). We acknowledge that the model may oversimplify *her* gene regulation in the zebrafish PSM; promoter analysis indicates that the *her1* promoter is negatively regulated by Her7 but not by Her1 (Gajewski et al., 2003). In our model (Fig. 5), *tortuga* could function either directly (to specifically target *her1*) or indirectly (as part of a general mRNA degradation pathway) to promote *her1* mRNA degradation. The auto-inhibitory model predicts that oscillations in the level of Her1 protein are required for proper transcriptional regulation of *her1* transcript. Consistent with this idea, a recent mouse knock-in study showed that replacing the endogenous Hes7 coding sequence with that encoding a more stable Hes7 protein disrupts *hes7* transcription and somite formation (Hirata et al., 2004). Because *her1* transcriptional oscillations are normal in *tor* mutant embryos, we suggest that the *tor* lesion does not significantly disrupt the oscillations of functional Her1 protein. The *tor* mutant phenotype highlights the importance of post-transcriptional mechanisms in regulating gene expression in the PSM. We anticipate that the molecular identification of *tortuga* will shed light on the mechanism of post-transcriptional regulation of Notch pathway transcripts during somitogenesis.

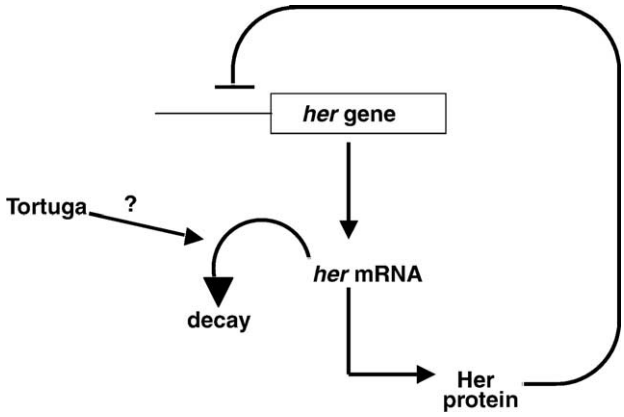

Fig. 5. A model for the function of the *tortuga*-encoded protein during zebrafish segmentation. Hes/Her autoinhibition has been proposed as a mechanism for generating oscillations of *her* gene expression in the vertebrate PSM. We propose that Tortuga acts to promote rapid *her1* mRNA degradation. Model adapted from Giudicelli and Lewis (2004).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.07.032.

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